Localization of the equine IgG-binding domain in the fibrinogen-binding protein (FgBP) of *Streptococcus equi* subsp. *equi*

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Fibrinogen-binding protein (FgBP, also termed SeM) is a cell-wall-associated anti-phagocytic M-like protein of the equine pathogen *Streptococcus equi* subsp. *equi*, and binds fibrinogen (Fg) and IgG. FgBP binds Fg avidly through residues located at the extreme N terminus of the molecule, whereas the IgG-binding site is more centrally located between the A and B repeats. FgBP binds equine IgG4 and IgG7 subclasses through interaction with the CH2–CH3 interdomain region of IgG-Fc, and possesses overlapping Fc-binding sites with protein A and protein G. In this study, FgBP truncates containing defined internal deletions were used to identify a stretch of 14 aa (residues 335–348) critical for IgG binding. Protein chimeras consisting of the non-IgG-binding α-helical coiled-coil M5 protein fused to FgBP sequences were used to identify a minimal equine IgG-binding domain consisting of residues 329–360. Competition ELISA tests suggested that IgG does not compromise Fg binding and vice versa.

INTRODUCTION

Streptococcus equi subsp. *equi* (herein referred to as *S. equi*) is the causative agent of strangles, a highly contagious, debilitating disease of the upper respiratory tract and associated lymph nodes of the horse (Timoney, 1993). *S. equi* is an obligate pathogen of the family Equidae, and is one of the most prevalent diseases of the horse, causing serious economic loss. With no adequate vaccine, prevention relies on prompt identification of infected horses, in combination with isolation and hygiene control measures (Waller & Jolley, 2007).

S. equi possesses many secreted and surface-associated proteins which bind host serum or matrix proteins and are likely to play roles in adhesion, invasion and evasion of the immune response (reviewed by Timoney, 1993, 2004; Harrington *et al.*, 2002; Waller & Jolley, 2007). These include two fibrinogen (Fg)-binding proteins (SzPSe and M protein). Of these, the M protein is considered to be an important virulence determinant (Meehan *et al.*, 1998, 2001; Timoney *et al.*, 1997). Fifteen alleles of the gene that encodes M protein have so far been identified (Kelly *et al.*, 2006; Waller & Jolley, 2007). M protein encoded by strain

TW and termed FgBP (fibrinogen-binding protein; gene allele 1) in our previous publications is almost identical to the prototype M protein (termed SeM) of strain CF32 (gene allele 2; Meehan *et al.*, 1998; Timoney *et al.*, 1997). The term FgBP will be retained in this publication to facilitate correlation with our previous publications and truncates (Lewis *et al.*, 2008a; Meehan *et al.*, 1998, 2000a, b, 2001, 2002).

FgBP possesses sequence and structural features in common with the cell-wall-associated M proteins of *Streptococcus pyogenes*, including a high proportion (over 60 %) of coiled-coil dimeric structure and Fg-binding ability (Fig. 1a). The region of FgBP involved in Fg binding is localized in the extreme N terminus of the protein, but requires downstream stabilizing coiled-coil structure (Meehan *et al.*, 2000a, b). There is convincing evidence that FgBP plays a significant role in resistance to phagocytosis and that the Fg-binding region is involved in this process (Boschwitz & Timoney, 1994a, b; Chanter *et al.*, 2000; Meehan *et al.*, 1998, 2001). The observed variability among the N termini of FgBP proteins from different *S. equi* strains has been exploited as a tool in epidemiological studies (Anzai *et al.*, 2005; Kelly *et al.*, 2006; Waller & Jolley, 2007).

FgBP additionally binds to the CH2–CH3 interdomain region of equine IgG (eqIgG)-Fc (Lewis *et al.*, 2008a;

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Abbreviations: Fg, fibrinogen; FgBP, fibrinogen-binding protein; HRP, horseradish peroxidase; eqFg, equine Fg; eqlgG, equine IgG.
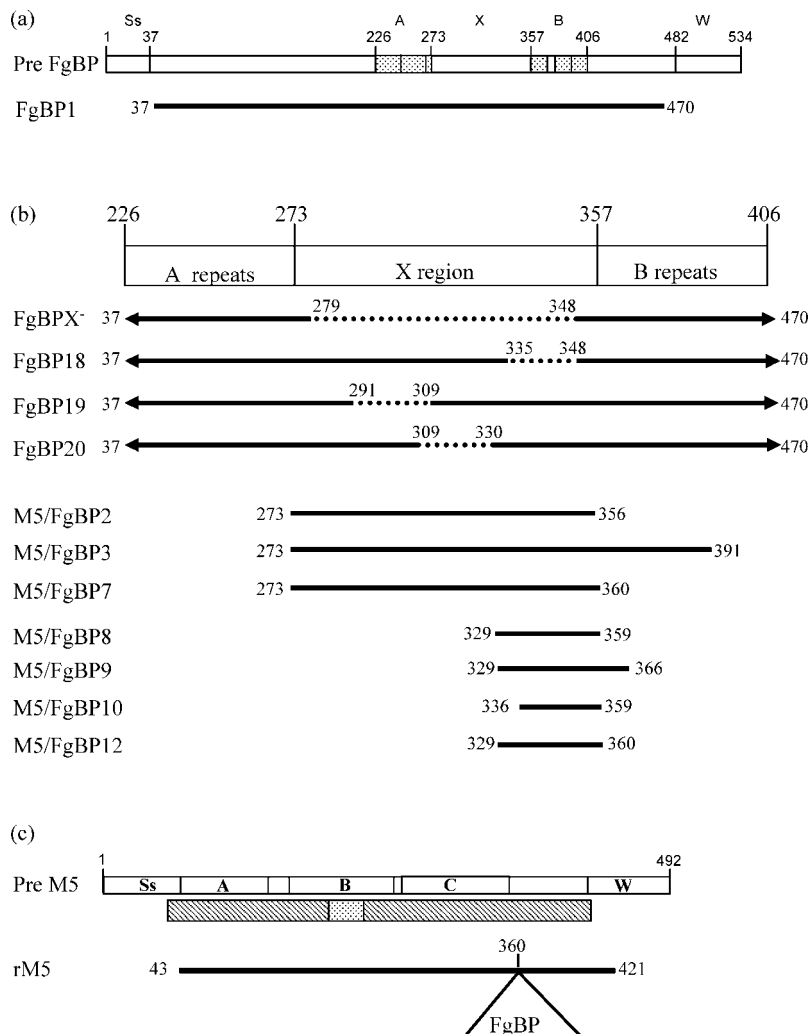
Fig. 1. Schematic diagram of (a) FgBP, (b) His-tagged recombinant FgBP truncates and M5FgBP chimeric proteins, and (c) M5 protein. (a) The positions of the signal sequence (Ss), A and B repeat domains, the X region between the A and B repeats, and the wall/membrane (W)-spanning region in unprocessed FgBP are indicated. Below the structure of pre-FgBP is a scaled representation of the recombinant His-tagged FgBP1 protein, which exhibits wild-type FgBP binding to both Fg and IgG. (b) Below a schematic diagram of the central region of FgBP are scaled representations of FgBP truncates constructed in this study. Dotted lines indicate the regions deleted. Also shown are scaled representations of the regions of FgBP which were used to construct M5FgBP chimeric proteins. (c) The positions of the signal sequence (Ss), the repeat regions (A, B and C) and the wall/membrane (W)-spanning domain of M5 protein are indicated. The box below indicates the regions of M5 predicted to have 90–100 % (hatched) and 22–62 % (dotted) α-helical coiled-coil structure. Also shown is a scaled representation of the recombinant M5 protein indicating the region into which FgBP sequences were inserted.

Meehan *et al.*, 2001). This is one of the few regions in IgG particularly suited for protein–protein interaction, and numerous bacterial, viral and host proteins bind to this region (Burton, 1985; DeLano *et al.*, 2000). Most bacterial immunoglobulin-binding proteins exhibit little or no sequence homology and are generally considered to have converged during evolution to bind overlapping sites in the interdomain region, with the observed diversity in immunoglobulin-binding profiles with respect to isotype, subclass and species being accounted for by the different binding interactions (Frick *et al.*, 1992; Goward *et al.*, 1993; Pleass *et al.*, 2001). The best studied IgG-binding proteins are protein A and protein G. Protein G and protein G-like proteins are responsible for much of the IgG binding by group C and group G streptococci (Björck *et al.*, 1987; Navarre & Schneewind, 1999), whereas IgG binding by *Staphylococcus aureus* is mediated by protein A and/or Sbi, a protein A-like protein (Atkins *et al.*, 2008; Moks *et al.*, 1986; Uhlén *et al.*, 1984). In *S. pyogenes*, immunoglobulin binding is mediated by three groups of M proteins encoded by the *emm*, *mrp* and *enn* genes.

Recently, Lewis *et al.* (2008a) characterized the immunoglobulin-binding properties of FgBP and showed that of the seven equine IgG subclasses, FgBP binds predominantly to IgG4 and IgG7. Together, IgG4 and IgG7 form the originally designated eqIgGb subclass, which is the most common IgG subclass in the serum and secretions of horses (Sheoran *et al.*, 1997). FgBP has been shown to require both the CH2 and the CH3 domain of IgG-Fc for full binding, the binding site exhibiting significant overlap with the Fc-binding sites for protein A and protein G (Lewis *et al.*, 2008a). Significantly, the interaction of FgBP with IgG is able to disrupt C1q binding and to inhibit the antibody-mediated classical complement pathway, suggesting another mechanism by which FgBP mediates immune evasion (Lewis *et al.*, 2008a).

Previous studies utilizing a series of FgBP truncates clearly showed that the binding of Fg and IgG requires distinct structural features (Meehan *et al.*, 2000a, b, 2001). IgG binding does not involve the extreme N terminus of FgBP required for Fg binding but rather a region spanning the

central portion of the protein between the A and B repeats (Meehan *et al.*, 2001). In this report, recombinant FgBP proteins possessing internal deletions and M5FgBP chimeric proteins were constructed and were employed to further define the IgG-binding site. Results suggest that IgG binding is mediated by a stretch of amino acids (329–360) adjacent to the B repeats. Within this 32-residue domain is a stretch of 14 aa shown to be critical for ligand binding.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* XL1-Blue (Stratagene) and plasmid pQE30 (Qiagen) were used for expression of recombinant FgBP and M5 proteins. *E. coli* strains were grown at 37 °C in LB medium supplemented where appropriate with ampicillin (100 μg ml^{-1}) and 1 mM IPTG. Plasmid pLZM5 was used as a template for PCR amplification of the *S. pyogenes* M5 gene (Ringdahl *et al.*, 2000; Johnsson *et al.*, 1998). *S. equi* subsp. *equi* strain TW genomic DNA and pQE30-FgBP1 were used as templates for PCR of *fbp*.

Recombinant DNA techniques. Genomic DNA was isolated from *S. equi* using the Edge BioSystems bacterial genomic DNA purification kit. Plasmid DNA was purified from *E. coli* using the Promega Wizard plasmid purification kit. DNA restriction digestions, ligations and transformations were carried out by standard methods (Sambrook *et al.*, 1989).

Expression and purification of recombinant proteins. FgBP1 was produced as previously described (Meehan *et al.*, 1998). Recombinant FgBP proteins containing internal deletions were constructed by amplification of *fbp* DNA fragments flanking the region to be deleted followed by their subsequent ligation in the expression plasmid pQE30 (Fig. 1b). pQE30-M5 was constructed by ligation of amplified DNA encoding amino acids 43–421 of the M5 protein into pQE30 predigested with *Bam*HI and *Xma*I. pQE30-M5FgBP recombinant plasmids were generated by (i) inverse PCR of pQE30-M5 DNA at nucleotide 1122 of the M5 gene using primers with *Sac*I and *Eco*RV restrictions sites and (ii) ligation of amplified *fbp* DNA fragments into amplified pQE30-M5 DNA. His-tagged recombinant FgBP and M5 proteins were purified from *E. coli* XL-1 Blue by metal chelate affinity chromatography, as described previously in detail (Meehan *et al.*, 1998, 2000a). Primers used in the construction of the FgBP/M5 recombinant proteins are listed in Table 1. Recombinant eqIgG subclasses were produced and purified as previously described (Lewis *et al.*, 2008b).

Biochemical procedures. SDS-PAGE was performed using either 12.5 or 7.5 % (w/v) polyacrylamide separating gels and a 4.5 % (w/v) polyacrylamide stacking gel. Samples were routinely heated for 3 min at 100 °C in Laemmli sample buffer prior to electrophoresis (Laemmli, 1970). Proteins were detected by staining with Coomassie brilliant blue. Molecular masses were determined from the relative mobilities of 15 standard molecular mass marker proteins (Gibco-BRL, BenchMark protein ladder). Protein concentration was estimated by the method of Bradford (1976) using BSA as a standard.

Immunological and affinity procedures. eqIgG-Fc affinity immunoblotting was performed as described previously (Meehan *et al.*, 2001) using horseradish peroxidase (HRP)-conjugated horse Fc (10 μg ml^{-1}; Jackson Immunoresearch). Fg-affinity blotting using HRP-labelled equine Fg (eqFg) was performed as described previously (Meehan *et al.*, 1998, 2000a). Prior to labelling with HRP, contaminating IgG was firstly removed from eqFg (Sigma Aldrich) using protein G agarose (Sigma Aldrich).

Table 1. PCR primers used to construct recombinant FgBP and M5 proteins

Primer*	Sequence (5′–3′)†
F1	cgcggatccaactctgaggttagtcgtacg
R1	atctgctgttttagctgcc
F2	ccgggatatcgaatcaggcaaacgtgaaattgc
R2	gcgcccgggttattgcttcaagcgattgatttc
F3	atcactgctgataaggctaaggc
R3	atctgtttgaagtgatgcaatggtg
F4	atccaaacagagctagaaaaagc
R4	ccgggatatctagactaccaacgttttc
F5	cgcggatccgccgtgactaggggtacaataaatg
R5	ccgggatatcaatttctgctaatttttgagc
F6	cggcgagctcaaattagctgctcttgaaaaac
R6	cgccccggggggtttgtgagtctgatgctttttgg
F7	cggcgatatcgcagctaaaacagcagaagc
R7	cggcgatatcgctgtttgcttcttctaaagc
F8	cggcgatatcgcatcacttcaaacagagctag
R8	cggcgagctcaatttcacgtttgcctgattc
F9	cggcgatatcgaaaaagctaagacagagcttg
R9	cggcgagctctaccttagcatctgatgctgc
R10	cagggagctcttgtagctcagcaatttc
R11	cagggagctctagctcagcaatttcacgttg
R12	cagggagctcagaagcatcttttttgttttttg

*The combinations of primers used in construction of recombinant proteins were: F1 with R1 and F2 with R2 (FgBPX^{-}); F1 with R3 and F2 with R2 (FgBP18); F1 with R4 and F3 with R2 (FgBP19); F1 with R5 and F4 with R2 (FgBP20); F5 with R6 (M5); F6 with R7 (pQE30-M5 inverse PCR); F7 with R8 (M5FgBP2); F7 with R9 (M5FgBP3); F7 with R10 (M5FgBP7); F8 with R11 (M5FgBP8); F8 with R12 (M5FgBP9); F9 with R11 (FgBP10); F8 with R10 (M5FgBP12).
†Restriction sites are underlined.

ELISAs to test binding to recombinant eqIgG4 and eqIgG7 were performed as previously described (Lewis *et al.*, 2008a). Plates (96-well) were coated with recombinant FgBP proteins (10 μg ml^{-1}), probed with eqIgG4 or eqIgG7 (0–20 μg ml^{-1}) in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7) containing 0.5 % (v/v) Tween-20 (PBS-T) followed by HRP-conjugated goat anti-mouse λ light-chain antibody (0.2 μg ml^{-1} in PBS-T; Bethyl Laboratories).

For competition ELISA tests, 96-well plates were coated overnight at 4 °C with FgBP (0.25 μg ml^{-1}), washed and then blocked with 5 % (w/v) non-fat milk powder in PBS-T containing 0.5 % (w/v) BSA. Plates were then co-incubated with 100 μl per well each of HRP-labelled eqFg (0.5 μg ml^{-1}) and serial dilutions (0–10 μg ml^{-1}) of unlabelled eqFg, eqIgG4, eqIgG7 or eqIgG5. Plates were incubated for 45 min at room temperature and then developed with Sure Blue HRP substrate (KPL).

RESULTS AND DISCUSSION

Residues 335–348 of FgBP are critical for IgG-Fc binding

We previously showed, using a panel of N-terminal and C-terminal truncates and truncates lacking the repeat domains, that residues between the A and B repeats of

FgBP (the X region in Fig. 1) are important for IgG-Fc binding (Meehan et al., 2001). In order to identify which residues within this domain were critical for IgG binding, His-tagged recombinant FgBP proteins possessing internal amino acid deletions were constructed and purified (Figs 1b and 2a). FgBP1 was used as a starting point in construction, since it possesses similar IgG-Fc binding to that of wild-type FgBP purified directly from S. equi (Fig. 1a; Meehan et al., 2001).

In the design of recombinant derivatives, care was taken to ensure that the proposed deletions did not affect predicted
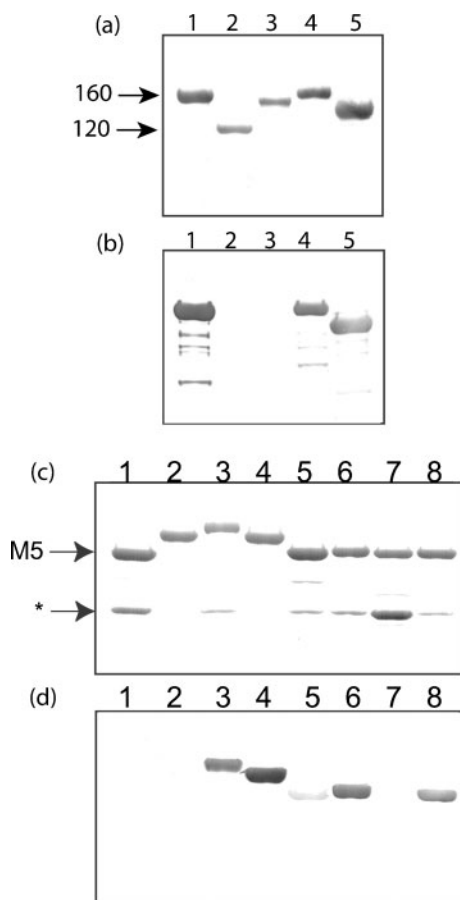

Fig. 2. SDS-PAGE and Western affinity blot analysis of purified His-tagged FgBP and M5FgBP proteins. (a) SDS-PAGE analysis of FgBP proteins. Lanes: 1, FgBP1; 2, FgBPX⁻; 3, FgBP18; 4, FgBP19; 5, FgBP20. Samples were analysed on a 7.5 % (w/v) polyacrylamide separating gel. The positions of the 160 and 120 kDa marker proteins are indicated. (b) An analogous Western affinity blot probed with HRP-conjugated eqIgG-Fc. (c) SDS-PAGE analysis of M5FgBP proteins. Lanes: 1, M5; 2, M5FgBP2; 3, M5FgBP3; 4, M5FgBP7; 5, M5FgBP8; 6, M5FgBP9; 7, M5FgBP10; 8, M5FgBP12. Samples were analysed on a 12.5 % (w/v) polyacrylamide separating gel. The positions of M5 and a degradation product (asterisk) seen with some of the recombinant proteins are indicated. (d) An analogous Western affinity blot probed with HRP-conjugated eqIgG-Fc.

α-helical coiled-coil structure as determined by the COILS program (Lupas et al., 1991; Lupas, 1996). Like FgBP1, these truncates lacked the signal sequence and the cell wall/membrane-spanning region. In addition, they lacked residues 279–348 that encompassed most of the X region between the A and B repeats (FgBPX⁻) or several shorter amino acid stretches within the X region that included residues 335–348 (FgBP18), residues 291–309 (FgBP19) and residues 309–330 (FgBP20; see Fig. 1b). Following analysis by SDS-PAGE, all purified proteins gave a single dominant band with an apparent M_r three to four times greater than that predicted from the sequence (see Fig. 2a). The aberrant mobility of FgBP and its recombinant truncates following SDS-PAGE is well documented (Meehan et al., 2000a, b).

Western affinity blots showed that the positive control (FgBP1), FgBP19 and FgBP20 all bound IgG-Fc, whereas FgBP18 and FgBPX⁻ did not (Fig. 2b). To place these observations on a more quantitative basis and to relate them to previous experiments, IgG-Fc binding was analysed using semiquantitative dot blots. Results confirmed that FgBP18 and FgBPX⁻ exhibited a dramatic (128-fold) reduction in binding to eqIgG-Fc and that FgBP19 and FgBP20 bound levels of eqIgG-Fc only slightly less (twofold to fourfold less) than that of FgBP1 (Fig. 3a). Furthermore, in ELISA tests, FgBP18 and FgBPX⁻ failed to bind recombinant eqIgG4 and eqIgG7 subclasses. In contrast, eqIgG4 and eqIgG7 showed half-maximal binding of 0.5 and 0.8 µg ml⁻¹ to FgBP1 and FgBP20, respectively (Fig. 4a, b). Analysis of the ability of these internally deleted FgBP truncates to bind Fg showed that FgBP18–20 bound the ligand with an avidity similar to that of FgBP1, whereas FgBPX⁻ (with a larger deletion) possessed a fourfold reduction in Fg binding (Fig. 3b). Together, these results strongly suggest that residues 335–348 are crucial for IgG binding but not for Fg binding.

Residues 329–360 of FgBP represent a minimal IgG-binding sequence

Identification of critical IgG-binding residues allowed the rational design of experiments to identify a minimal IgG-binding sequence. Previously, circular dichroism and thermal stability analysis provided convincing evidence that the C terminus of FgBP encompassing the IgG-Fc-binding region contributes markedly to the thermal stability of FgBP and that coiled-coil structure may be required for ligand binding (Meehan et al., 2002). Therefore, to maintain the structural stability of short FgBP sequences and to minimize any loss in ligand binding resulting from destabilization of coiled-coil structure, M5FgBP chimeric proteins were constructed. M5 protein was employed as it possesses a high probability of α-helical coiled-coil structure over the majority of the protein. M5 binds human Fg using residues in its B repeat region, but importantly does not bind IgG (Fig. 1c; Ringdahl et al., 2000). To construct M5FgBP chimeric proteins, the region
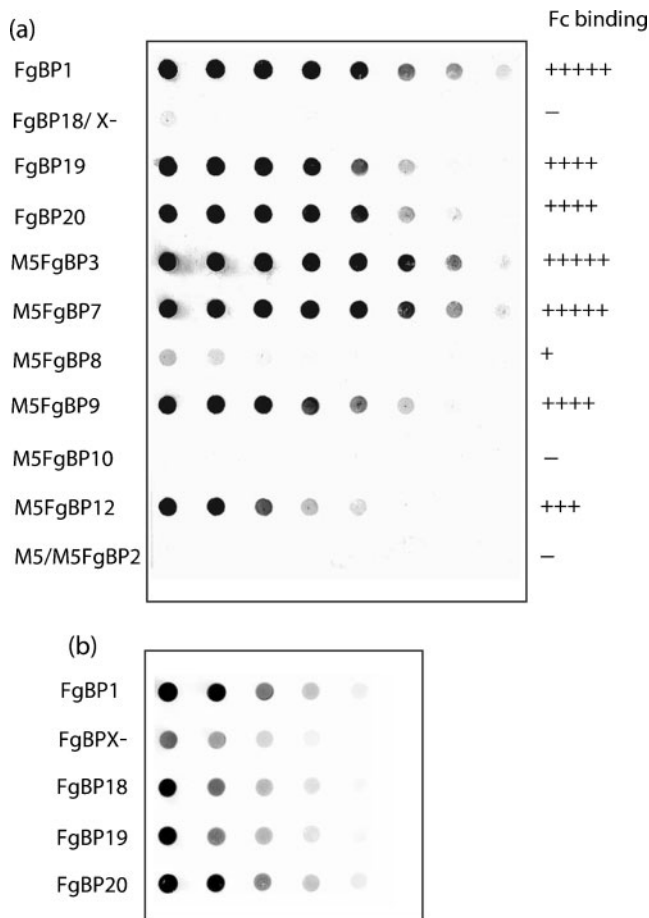
Fig. 3. (a) eqIgG-Fc and (b) Fg affinity dot blot analysis of undenatured FgBP truncates. Sample identity is indicated at the side of the blots. (a) The first well in each row of doubling dilutions contains 75 pmol of truncate and samples were probed with HRP-conjugated eqIgG-Fc. FgBP truncates X⁻ and 18 gave identical negative results, as did M5, M5FgBP2 and M5FgBP10. IgG-Fc binding abilities are indicated to the right. Wild-type binding (equivalent to wild-type FgBP) is indicated by +++++, and twofold, fourfold to eightfold, 64-fold and 128-fold less binding than wild-type binding is indicated by ++++, +++, + and −, respectively. (b) The first well in each row of doubling dilutions contains 9.4 pmol of truncate. Samples were probed with HRP-conjugated eqFg.

of M5 DNA encoding the first 379 aa of mature M5 was first cloned into the expression vector pQE30. This recombinant plasmid expressed a truncated M5 protein which lacked the signal sequence and wall/membrane region, and was used as template for insertion of *fbp* DNA. The resultant M5FgBP recombinant chimeras possessed FgBP sequences inserted between amino acids 360 and 361 of M5 protein (Fig. 1c), and included constructs that contained the X region (M5FgBP2) and the X region plus some downstream sequence (M5FgBP3 and M5FgBP7). Also constructed were several recombinants covering the region shown to be critical for IgG binding (M5FgBP8/9/

10/12; see Fig. 1b). Computer predictions showed that the coiled-coil structures of M5FgBP3 and M5FgBP8–10 were unchanged from that predicted for wild-type FgBP (Lupas *et al.*, 1991; Lupas, 1996). These constructs were designed so that when fused into the M5 protein, FgBP residues would have the same predicted position in the heptad of coil-coil structure as in the native protein (Fig. 5a). Thus, Ala329 and Glu336 are at **b** positions, and Leu359 and Ser366 are at **d** positions. At either end of inserted FgBP sequences, positions **g** and **a** are provided by Asp and Ile, and positions **e** and **f** are provided by Glu and Leu from introduced restriction sites. For M5FgBP2, there is a predicted three-residue deletion (stutter) in the heptad repeat after Gly352 (**d** position) to Lys353 (**a** position). For M5FgBP7 and M5FgBP12, possession of Gln360 results in a predicted extra residue at the **e** position at the end of the FgBP insert. Deletions/insertions of these types can cause local aberrations in coiled-coil structure (Mason & Arndt, 2004). However, all purified M5FgBP fusion proteins (Figs 1b and 2c) bound human Fg, suggesting that no gross structural changes in M5 had occurred as a result of inclusion of FgBP sequences (data not shown).

Western IgG-affinity blotting experiments showed that M5, M5FgBP2, M5FgBP8 and M5FgBP10 bound eqIgG-Fc weakly or not at all and that the remaining chimeric proteins (M5FgBP3, 7, 9 and 12) bound detectable levels of eqIgG-Fc (Figs 1b and 2d). IgG-affinity dot blots performed using undenatured recombinant chimeras confirmed and extended these observations (Fig. 3a). M5FgBP3 and M5FgBP7 bound wild-type levels of IgG-Fc, whereas M5FgBP9 (possessing residues 329–366 of FgBP) exhibited a slightly reduced (twofold) binding similar to that observed for FgBP19/20. M5FgBP12 (possessing residues 329–360 of FgBP) showed a further two- to fourfold reduction in binding. In contrast, M5FgBP2, M5FgBP8 (possessing residues 329–359 of FgBP) and FgBP10 (possessing residues 336–359 of FgBP) exhibited significantly reduced binding to IgG-Fc (Fig. 3a). ELISAs performed using recombinant eqIgG4 and eqIgG7 further revealed that: (a) M5FgBP3 bound similar levels of IgG to that of FgBP1; (b) M5FgBP9 and M5FgBP12 bound levels which were reduced compared with FgBP1 and somewhat similar to those observed for FgBP20; (c) M5 and M5FgBP10 did not bind either IgG subclass.

Taken together, these data suggest that the smallest region of FgBP that still retains significant IgG binding corresponds to residues 329–360 and encompasses the 14-residue critical domain (residues 335–348) identified through the use of recombinant truncates. Gln360 appears to be crucial for IgG binding, since M5FgBP8 (residues 329–359) and M5FgBP2 (residues 273–356) show significantly reduced binding to IgG-Fc compared with M5FgBP12 (residues 329–360) and M5FgBP7 (residues 273–360). Residues 329–360 impart significant but not full wild-type binding to the M5FgBP chimera. Available evidence suggests that upstream residues are necessary to achieve this. Firstly, M5FgBP7, which possesses residues 273–360, bound wild-type levels of IgG. Furthermore,
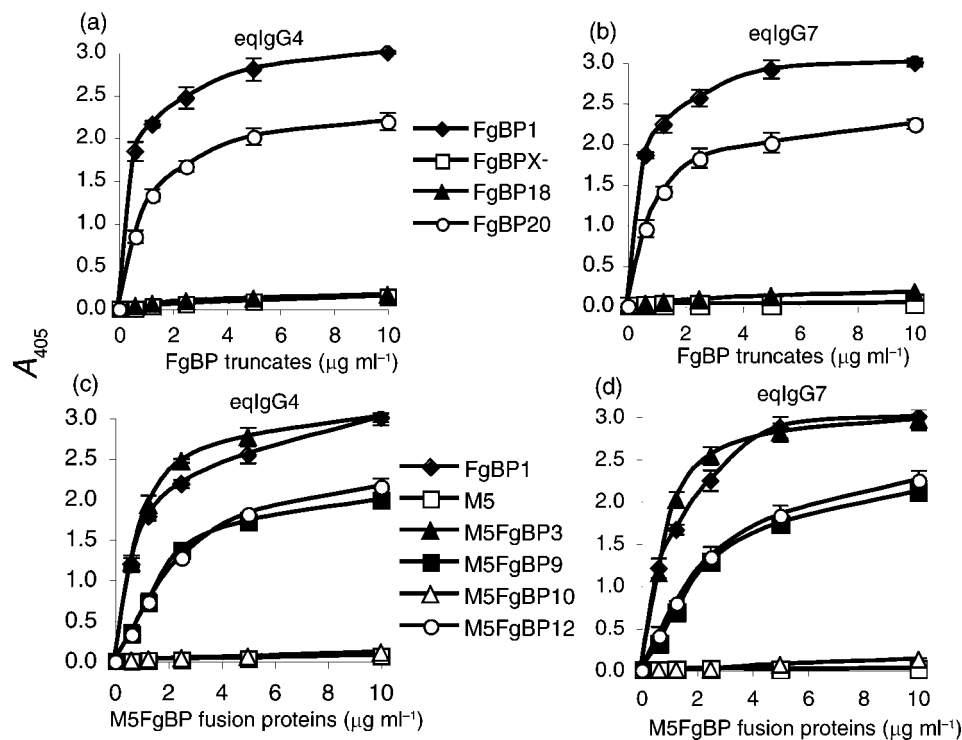
Fig. 4. ELISA analysis of the interaction of recombinant eqIgG4 and eqIgG7 with FgBP truncates (a, b) and M5FgBP chimeric proteins (c, d). The means (±SE) of three independent experiments are shown.

FgBP19 and FgBP20, which both possess deletions upstream of the 'critical' domain, exhibited a reduction in binding to eqIgG similar to that observed for the M5FgBP9/12 (Figs 3a and 4a, b). These upstream residues may provide some initial contact binding or play other structural role(s) which facilitate ligand interaction for the M5FgBP3 and M5FgBP7 chimeras. A similar phenomenon may account for the significant reduction in IgG binding observed for truncate FgBP17, which lacks the first 182 residues of mature FgBP (Meehan *et al.*, 2001).

The 32-residue IgG-binding domain possesses a strong probability of coiled-coil dimer formation. However, to date there is poor understanding of the structural parameters of streptococcal coiled-coil M or M-like proteins that are involved in IgG binding. Nonetheless, provided there is no local unwinding of the coiled-coil structure of FgBP, residues occupying solvent exposed-positions (i.e. **b**, **c**, **e**, **f** and **g**) are more likely to be involved in direct binding to IgG than non-polar residues buried at the interface of the two helices (**a** and **d** positions; Fig. 5a).

The IgG-binding domain of FgBP exhibits similarity to other coiled-coil and IgG-binding proteins

With a view to identifying possible conserved residues or motifs involved in IgG binding to FgBP, BLAST homology searches were conducted using the C-terminal region (residues 273–482) of FgBP as the target. Proteins exhibiting homology with the IgG-binding domain of FgBP included several streptococcal proteins with predicted coiled-coil structure, some of which have been documented to bind IgG (Fig. 5b). These included SzM of *S. equi* subsp. *zooepidemicus*, Spa of *S. pyogenes* strain 18 and DemA/B of *Streptococcus dysgalactiae*, as noted elsewhere (Dale *et al.*, 1999; Kelly *et al.*, 2006; McLellan *et al.*, 2001; Vasi *et al.*, 2000). Others included (A repeats of) the Mrp/FcrA protein family of *S. pyogenes* and (repeated residues in) Sez 1778 of *S. zooepidemicus* strain MGCS10565 (Beres *et al.*, 2008; O'Toole *et al.*, 1992). Alignments of the above proteins revealed two conserved lysines corresponding to Lys337 and Lys353 of FgBP. Lys353 is followed by the sequence REIAEL, present as conserved substitutions in all proteins except Sez, which possesses Lys instead of Leu. Other residues in FgBP with conserved substitutions in homologous proteins include Glu334, Glu341, Ala343, Ileu349 and Ser351 present in or adjacent to the critical domain.

The ligand-binding properties of the proteins indicated in Fig. 5 have only been established for FgBP, DemA and the Mrp proteins, all of which bind Fg and IgG. Significantly, the IgG-binding domain of FgBP aligns with domains within Mrp and DemA previously implicated in IgG binding (Heath *et al.*, 1990; Vasi *et al.*, 2000). Although the ligand-binding properties of SzM, Spa and Sez 1778 are
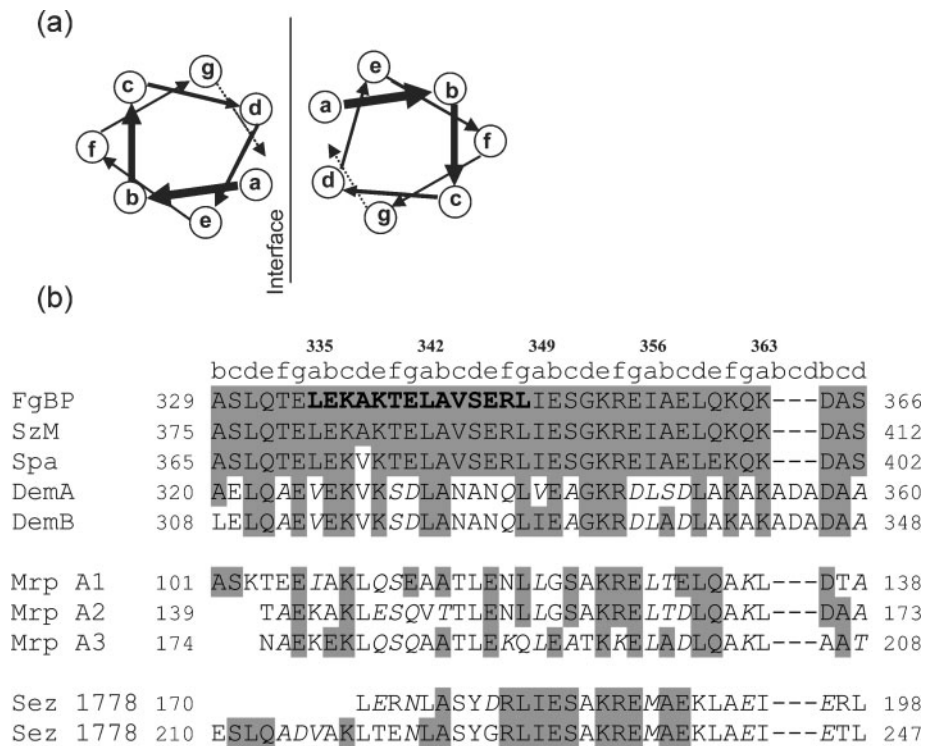
Fig. 5. Helical wheel representation of a dimeric coiled coil (a) and CLUSTAL W alignment of amino acids 329–366 of FgBP with relevant positions in SzM, Spa, DemA, DemB and repeated sequences in Mrp and Sez 1778 (b). (a) Coiled-coil dimers consist of a heptad repeat pattern (**a–g**). **a** and **d** are typically hydrophobic core residues present at the interface of the two helices and are involved in dimer stabilization, whereas the remaining residues are generally hydrophilic. (b) Residues in FgBP shown to be critical for IgG binding are in bold type. Above the protein alignment are the predicted positions (**a–g**) of the amino acid residues in the heptad repeat of their coiled coil and the amino acid numbers of FgBP residues corresponding to the **a** position of heptad repeats. The Dem proteins possess a three-residue (**bcd**) insert in the heptad, which is indicated. Conserved residues are shaded grey and conserved substitutions indicated in italic type. The gene accession numbers for Spa, Sez 1778, DemA, DemB and Mrp4 are AAD42939, CPOO1129, CAB65411, CAB65413 and AAA26930, respectively.
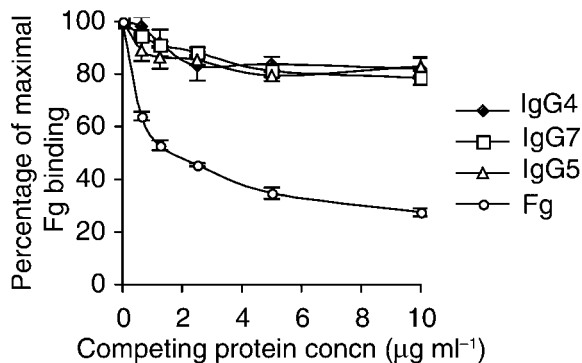

Fig. 6. ELISA of competition for binding to immobilized FgBP between HRP-labelled eqFg and increasing concentrations of unlabelled eqFg, recombinant eqIgG4, eqIgG7 or eqIgG5. The means (±SE) of three independent experiments are shown.

unknown (Beres *et al.*, 2008; Dale *et al.*, 1999; McLellan *et al.*, 2001), it seems highly likely that Spa and SzM are capable of binding IgG given the virtual identity of their C-terminal domains to residues 255–534 of FgBP (95 and 98 % identity, respectively; Dale *et al.*, 1999; Kelly *et al.*, 2006; McLellan *et al.*, 2001) and that this accounts for the reported anti-phagocytic properties of Spa (Dale *et al.*, 1999).

FgBP does not show homology to the proposed EQ-rich IgG3- and IgG1234-binding domains present in the class II group of M proteins and protein H of *S. pyogenes* (Bessen *et al.*, 1997; Pack *et al.*, 1996), nor does it show sequence homology to the IgG-binding domains of protein A or protein G. Despite the fact that all three proteins possess (some) α-helical structure in their binding domains, available evidence indicates that the strategy used by FgBP for binding IgG-Fc differs from the mode of interaction adopted by protein A and protein G. However, competition ELISAs and site-directed mutagenesis studies show that the FgBP-binding site on IgG

overlaps with the binding sites of protein A and protein G and involves residues in the Leu251–Ser254 and Leu432–Tyr436 loops of IgG that interact with protein A and/or protein G (Lewis *et al.*, 2008a). It is known that acidic and charged residues play important roles in the interaction between protein A/G and IgG-Fc (Sauer-Eriksson *et al.*, 1995). One of the most critical interactions for protein G is the one between the Leu251–Ser254 loop of IgG-Fc and Glu27, the carboxylate of Glu27 being held in position by hydrogen bonding to the adjacent Lys31 of protein G (Sauer-Eriksson *et al.*, 1995; Sloan & Hellinga, 1999). It has not escaped our notice that within the critical domain and adjacent to each other on the coiled-coil dimeric model structure of FgBP are residues Glu341 (**g** position), Lys337 (**c** position) and Lys339 (**e** position), which may perform analogous roles. However, it should be acknowledged that oppositely charged residues commonly occur in **e** and **g** positions of coiled-coils, providing inter-helical stability (Mason & Arndt, 2004).

IgG binding does not inhibit Fg binding

In the current study, we have shown that IgG binding is critically dependent on a region (residues 335–348) distinct from the N-terminally located Fg-binding domain (Meehan *et al.*, 2000a) and that deletion of the former region does not cause a significant reduction in Fg binding (Fig. 3b). In order to assess whether IgG binding could compromise Fg binding, competition ELISA tests were performed (Fig. 6). These tests showed that binding of eqFg to FgBP was slightly reduced in the presence of eqIgG4 and eqIgG7. However, this decrease was no greater than that shown in the presence of eqIgG5 (Fig. 6) or eqIgG3 (data not shown), both of which are eqIgG subclasses known to lack FgBP binding (Lewis *et al.*, 2008a). In contrast, unlabelled eqFg could significantly compete with HRP–eqFg for binding and HRP–eqFg binding was inhibited in a concentration-dependent manner by 70 %. Reverse competitive ELISAs showed that increasing doses of unlabelled Fg did not inhibit IgG binding (data not shown). Furthermore, preliminary surface plasmon resonance assays supported these findings and showed that Fg and IgG can bind simultaneously to FgBP (M. J. Lewis, unpublished observation).

Concluding remarks

An IgG-binding domain of FgBP has been identified which shows homology with regions present in other streptococcal coiled-coil proteins. Like FgBP, some of these proteins (Mrp and DemA) bind Fg and IgG. Future site-directed mutagenesis studies targeting conserved residues within this domain should allow identification of residues that are crucial for IgG binding. The IgG-binding domain of FgBP is located in a region of the protein different from that of the Fg-binding domain, and ELISA studies have indicated that binding of the two ligands is not competitive. Having defined the ligand-binding domains of FgBP, it is apparent

that the protein can be separated into two halves, the N-terminal Fg-binding region that is variable both within *S. equi* and among sub(species) (Anzai *et al.*, 2005; Chanter *et al.*, 2000; Kelly *et al.*, 2006) and the conserved C-terminal region that harbours the IgG-binding domain. The conservation of this latter region suggests that it is not a target for strong immune or host-specific pressure. Other workers have suggested that acquisition and/or modification of the N-terminal region of FgBP was a key event in the evolution of *S. equi* from an ancestral *S. zooepidemicus* strain (Kelly *et al.*, 2006; Waller & Jolley, 2007). Whether this is solely related to its Fg-binding function or to another as yet unknown function remains to be determined.

FgBP is clearly an important anti-phagocytic protein of *S. equi* able to circumvent the alternative and classical complement pathways through its ability to bind Fg and IgG (Boschwitz & Timoney, 1994a, b; Chanter *et al.*, 2000; Meehan *et al.*, 2001; Lewis *et al.*, 2008a). Perhaps full resistance to phagocytosis is through cooperation between the two ligand-binding functions, as proposed for M22 protein, which binds C4b-binding protein and IgA-Fc (Carlsson *et al.*, 2003). Certainly, the identification of IgG- and Fg-binding sequences of FgBP should allow the rational construction of *S. equi* mutants of value in determining the relative contribution to *S. equi* virulence of FgBP-mediated Fg/IgG binding and of other *S. equi* Fg- and IgG-binding proteins (e.g. EAG and SzPSe) (Flock *et al.*, 2004; Timoney *et al.*, 1997).

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